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Remarkable enhancement in the DNA-binding ability of C2-fluoro substituted pyrrolo[2,1-*c*][1,4]benzodiazepines and their anticancer potential

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ABSTRACT

C2-Fluoro substituted DC-81, and its dimers that comprise of two C2-fluoro substituted DC-81 subunits tethered to their C8-position through simple alkane spacers as well as piperazine moiety side-armed with symmetrical alkyloxy spacers have been designed and synthesized. These fluoro substituted pyrrolo[2,1-*c*][1,4]benzodiazepines have shown remarkable DNA-binding ability and most of them possess promising anticancer activity, having GI_{50} values in micromolar to nanomolar concentration range. DNA thermal denaturation studies show that some of these compounds (**14a–c** and **15**) increase the ΔT_m values in the range of 28.9–38 °C, and this is further confirmed by the restriction endonuclease studies. This study illustrates the importance of introducing fluoro substitution at the C2-position apart from the incorporation of a piperazine ring in between the alkyloxy linker for enhancement of the DNA-binding ability in comparison to DSB-120 and SJG-136 (ΔT_m = 10.2 and 25.7 °C). Moreover, the variations in the DNA-binding ability with respect to fluoro substitution in this class of dimers has been investigated by molecular modeling studies. Some representative C2-fluoro substituted dimers (**8a** and **14a**) have also exhibited significant anticancer activity in the 60 cancer cell line assay of the National Cancer Institute (NCI).

1. Introduction

Cancer is one of the most feared diseases in the modern society. A variety of approaches have been taken to cancer chemotherapy, and many antitumour drugs have been developed for clinical use, but cancer still remains one of the leading causes of human mortality. In the field of chemotherapeutic drugs, the search for new, more active, more selective and less toxic compounds is still very intense, and new promising anti-cancer approaches are being tested.[1,2]

Fluorinated organic molecules are known to perform a wide range of biological functions and fluorinated anticancer agents have become a focus in the development of new therapies for cancer. An increasing number of fluorinated antitumour agents have now become available for cancer treatment.[3] The importance of organo-fluorine compounds in medicinal chemistry continues to stimulate interest in the origins of the more cryptic effect of fluorine introduction. The development of pharmacological agents that are able to counteract the mechanisms of drug resistance in oncology has remained a major goal for the past two decades. Recently, a number fluoro based antiviral and antitumour agents have been developed.[4,5] For example 2′-difluoro-modified gemcitabine[6,7] exhibits potent antitumour activity and is clinically being used for the treatment of various solid tumors. Moreover, 5-fluorouracil (5-FU) has also been used extensively in the treatment of skin cancer and a variety of solid tumors such as breast, colorectal and gastric cancers.[8,9] Recently a series of C2-fluorinated PBDs have been synthesized and screened for cytotoxicity (IC_{50}) against a number of cancer cell lines,[10] in which fluorine substitution plays a key role for their biological activity.

DNA interstand cross-linking agents that interact within the minor groove constitute an important class of antitumour drugs.[11] There has been considerable interest in the design and development of DNA interactive ligands that are capable of binding to DNA in a sequence selective manner.[12] Pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) dimers are synthetic sequence-selective interstand DNA minor groove cross-linking agents developed from the monomeric anthramycin family of naturally occurring antitumour agents[13] like DC-81 (**1**), tomamycin (**2**) and anthramycin (**3**). These are tricyclic molecules that possess a chiral center at their C11a position, which provides them with a right-handed twist in the minor groove of DNA. Furthermore, an electrophilic imine carbinolamine moiety (at the N10-C11 position) allows the PBDs to bond covalently to guanine at the N2 position that results for their biological activity.[14] It is interesting to note that these C-ring modified PBDs can offer not only greater differential thermal stabilization of DNA duplex but could also significantly enhance the kinetic reactivity during covalent adduct formation. PBD monomer natural products

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with unsaturation at the C2-position are known to possess better biological activity than their C2-saturation derivatives. One explanation for this is that C2-*exo*-unsaturation lead to lower electrophilicity at N10-C11 position.[15,16] This may allow greater availability of the target DNA sequence due to a lower level of deactivation by cellular nucleophiles. In this context, a PBD dimer DSB-120 (**6**) has been synthesized by Thurston and co-workers[17] as a highly efficient DNA interstrand cross-linking agent. Similarly, C2/C2′ *exo*-unsaturated C8-linked PBD dimers have also been synthesized as efficient cross-linking agents[18] with much higher DNA-binding affinity and cytotoxicity, for example, SJG-136 (**9**). Moreover, recently mixed imine–amide and imine–amine PBD dimers (**11**) have been designed and synthesized in this laboratory with a view to understand the contributions from the non-covalent interactions by one of the subunit in these dimers. It has been observed that by incorporation of a non-covalent component is interestingly and surprising, as the DNA-binding affinity is significantly enhanced in such mixed type of PBD dimers.[19,20]

During the last decade, many piperazine derivatives have been synthesized as useful chemotherapeutic agents for various diseases. Bis-1,4-dialkyl-piperazines have been extensively investigated and are reported as antibacterial[21] as well as antineoplastic agents.[22] Recently, soluble cationic trans-diamine dichloroplatinum(II) complexes with piperazine ligands have been prepared that exhibit significant cytotoxic activity against cisplatin resistant ovarian cancer cells.[23] Moreover, the main objective to incorporate, a piperazine moiety is not only to improve the bioavailability but also DNA-binding ability in case of PBD dimers. Therefore, based on these observations and our earlier findings on the newly designed PBDs particularly with a fluorine substitution,[24–26] we wish to report the synthesis of C2-flouro substituted analogs of DC-81 and their dimers that have been linked through simple alkane spacers (**8a–c**) as well as through the piperazine side-armed with symmetrical alkane spacers (**13a–c**, **14a–c** and **15**) with a view to explore their DNA-binding ability and cytotoxicity. Some representative members of the PBD monomers and dimers with or without fluoro substitution have been illustrated in Figure 1.

2. Results and discussion

2.1. Chemistry

The synthesis of C2-difluoro substituted analogs of DC-81 (**4** and **5**) has been carried out by employing commercially available *trans*-4-hydroxyproline (**16**), as one of the starting material which is N-protected by Boc via its methyl ester to give compound **18** in quantitative yield.[27] The oxidation of 2-hydroxy group of compound **18** with trichloroisocyanuric acid and TEMPO gives the key precursor with C2-ketone (**19**) in excellent yield.[28] This has been converted to its C2-difluoro compound **20** employing DAST in dichloromethane.[29] This upon deprotection of Boc provides the required precursor methyl-(2*S*)-4,4-difluoropyrrolidine-2-car-

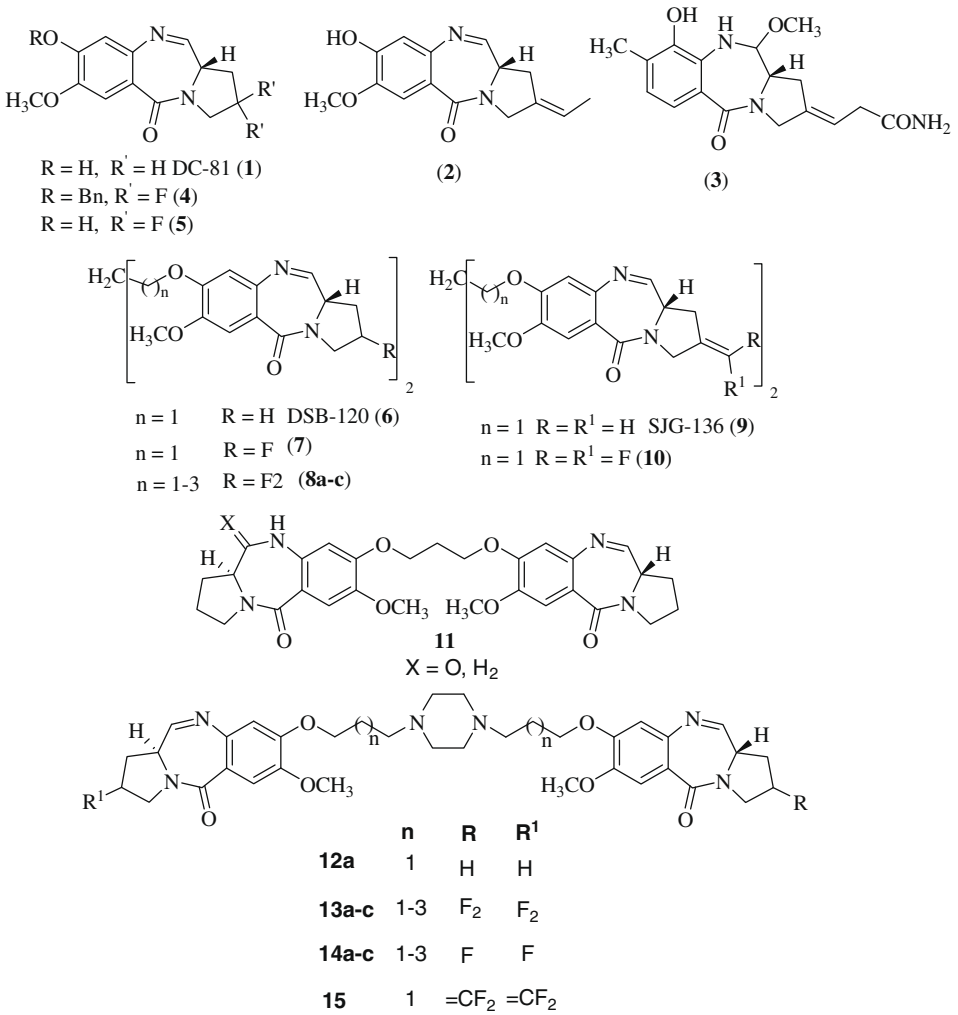
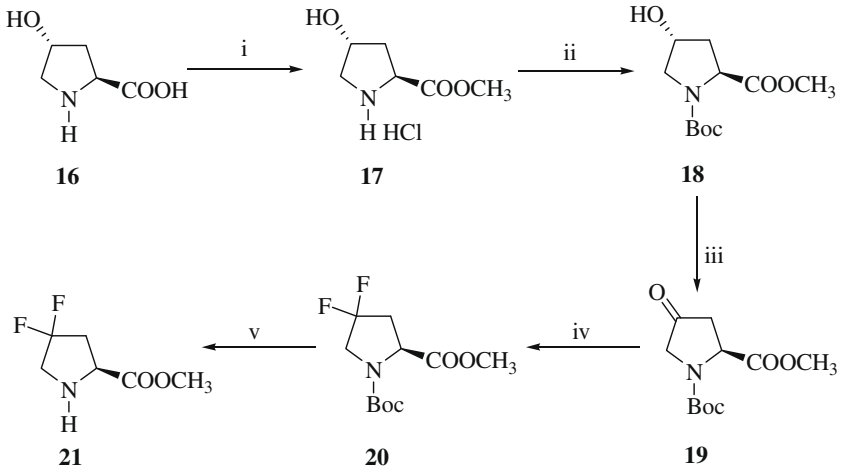


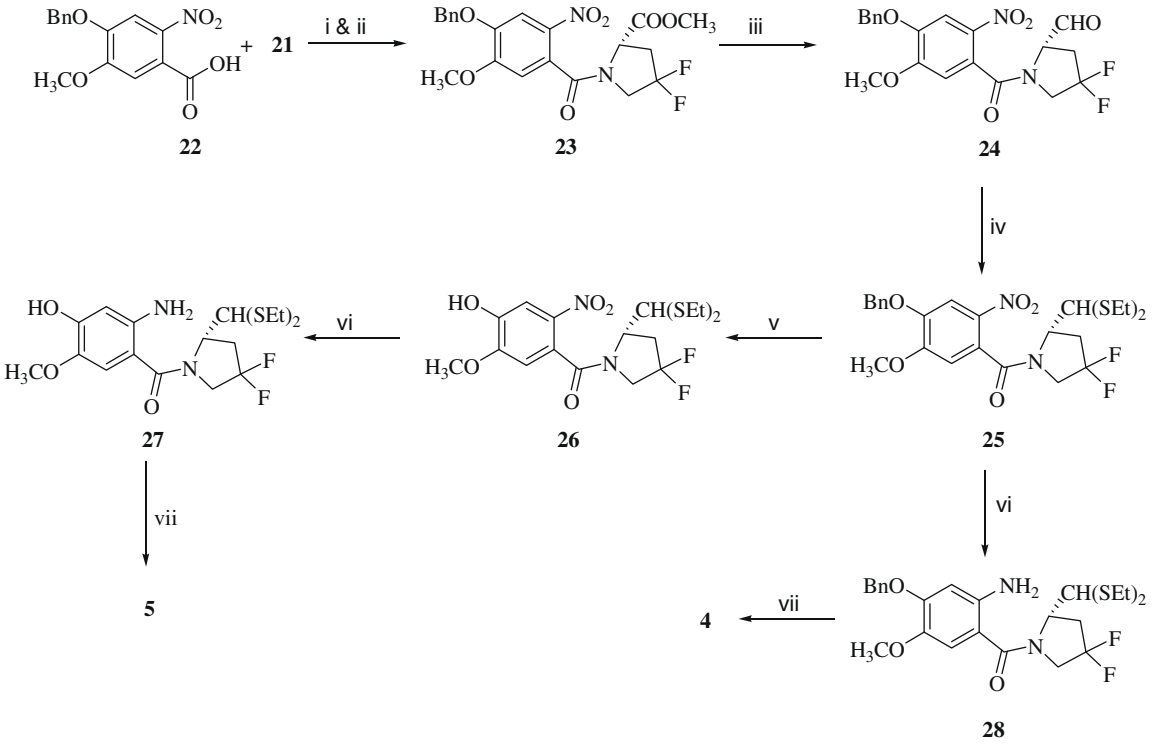
Figure 1. Chemical structures of PBD monomers and its dimers.

boxylate (**21**). The other precursor 4-benzyloxy-5-methoxy-2-nitrobenzoic acid (**22**) has been prepared by the previously reported methods[30] and is coupled to compound **21** via its acid chloride to give methyl-(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxylate (**23**). This up on reduction with DIBAL-H gives the corresponding aldehyde (**24**), which upon EtSH protection (**25**) followed by deprotection of benzyl group by using EtSH–$BF_3{\cdot}OEt_2$ provides the key intermediate **26**. Subsequent reduction of the intermediates **25** and **26** with tin chloride provides the amino thioacetal compounds **27** and **28**. These upon deprotective cyclization afford the target C2-difluoro substituted analogs of DC-81 (**4**, **5**) as shown in Schemes 1 and 2.

The synthesis of C2/C2′-difluoro substituted PBD dimers[31] has been carried out by the etherification of (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) with dibromoalkanes in acetone and K_2CO_3 to provide the nitro dimers **34a–c**. These upon reduction with tin chloride in methanol afford the corresponding amino diethylthioacetals **35a–c**. Deprotection of these amino thioacetals by employing $HgCl_2$–$CaCO_3$ provides the desired target products **8a–c** in overall 55–60% yields. However, this deprotection by employing bismuth triflate[32] in a biphasic system gives the products in yields ranging from 65% to 70% as illustrated in Scheme 3. In the later procedure the products are devoid of the trace impurities of mercuric salts and the purification process is simple.

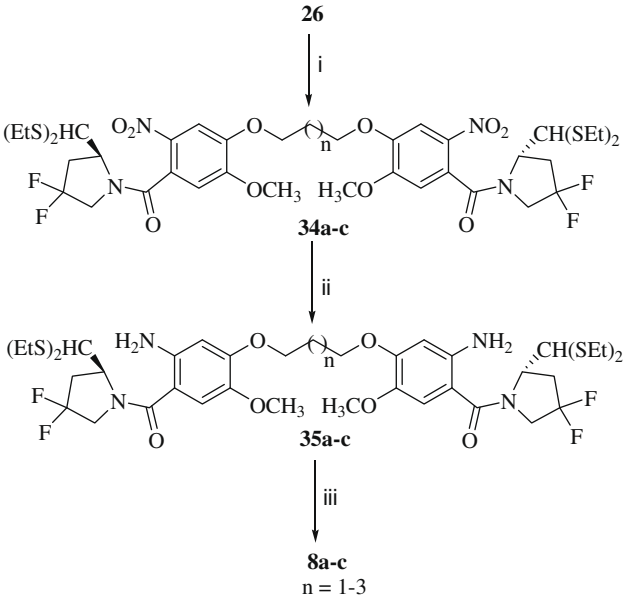


Scheme 1. Reagents and conditions: (i) $SOCl_2$, MeOH, rt; (ii) Boc_2O, 2 N NaOH, THF–H_2O, (2:1), rt, overnight; (iii) trichloroisocyanuric acid, TEMPO, CH_2Cl_2, 15 min; (iv) DAST, CH_2Cl_2, rt, 12 h; (v) TFA, CH_2Cl_2, 8 h.



Scheme 2. Reagents and conditions: (i) $SOCl_2$, DMF, benzene, rt, overnight; (ii) Et_3N, THF, H_2O, 0 °C, 1h; (iii) DIBALH, CH_2Cl_2, −78 °C, 1 h; (iv) EtSH–TMSCl, CH_2Cl_2, 16–18 h; (v) EtSH–$BF_3{\cdot}OEt_2$, CH_2Cl_2; (vi) $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 2 h; (vii) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O (4:1), 8 h.

Synthesis of C2/C2′-difluoro substituted PBD dimers[33] that are linked through piperazine a moiety (**13a–c**) has been carried out by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) as the starting material. This upon monoalkylation by reacting with dibromoalkanes in the presence of K_2CO_3 in dry acetone affords (2*S*)-*N*-[4-(*n*-bromoalkyl)oxy-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**29a–c**). The dimerization of **29a–c** has been carried out by employing piperazine in the presence of K_2CO_3 in refluxing dry acetonitrile to provide the dimer nitro precursor 1,1′-{[1,4-di(alkane-n-diyl)hexahydropiperazine]dioxy-bis[(11a*S*)-7-methoxy-2-nitrobenzoyl-(4,4-difluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (**36a–c**). These nitro dimers have been reduced with tin chloride in methanol to give the corresponding amino diethylthioacetal dimers (**37a–c**). Finally deprotection of the thioacetal group employing the literature procedure[34] affords the desired target compounds in good yields (60–65%) as shown in Scheme 4. However, the procedure employing bismuth triflate provides the products that are devoid of the possible trace impurities of mercuric salts and thereby simplifying the final purification procedure.



Scheme 3. Reagents and conditions: (i) dibromoalkanes, K_2CO_3, acetone, reflux, 48 h; (ii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 2 h; (iii) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O (4:1) or $Bi(OTf)_3{\cdot}xH_2O$, CH_2Cl_2–H_2O, 3–8 h.

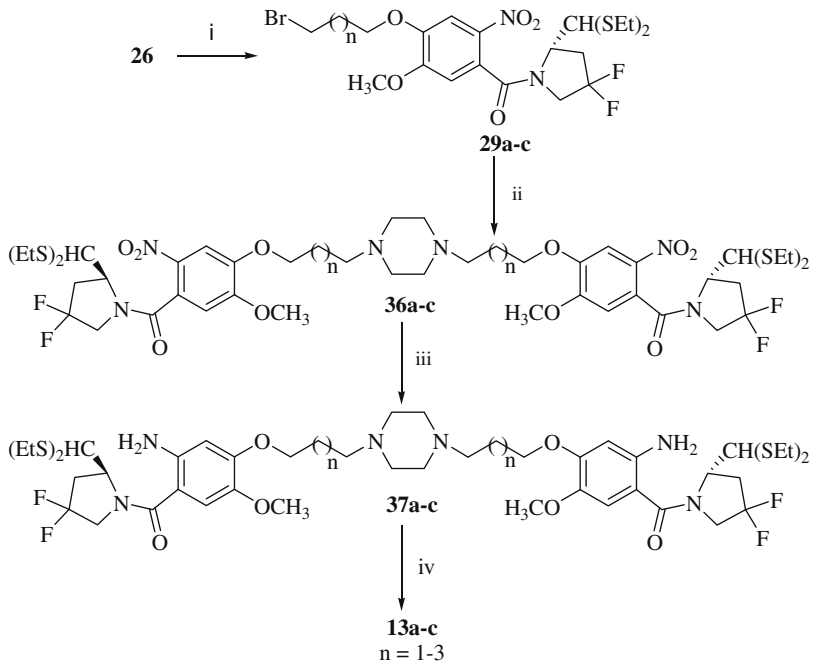
Similarly synthesis of C2-flouro substituted PBD dimers[33] that are linked through a piperazine moiety (**14a–c**) has been carried out by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4-fluoropyrrolidine-2-carboxaldehyde diethyl thioacetal (**30**) as the starting material, which is obtained by the reported method.[25] A four-step pathway provided the final assembly of the desired target compounds **14a–c** in the above conventional manner (Scheme 5).

The synthesis of C2-*exo*-difluoromethylidine substituted PBD dimers linked through piperazine moiety (**15**) has been carried out by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4-difluoromethylidenepyrrolidine-2-carboxaldehyde diethylthioacetal (**32**). This starting material has been prepared by the previously reported method.[26] A similar four-step pathway achieved the final assembly of the desired target compound **15** in the above similar manner (Scheme 6).

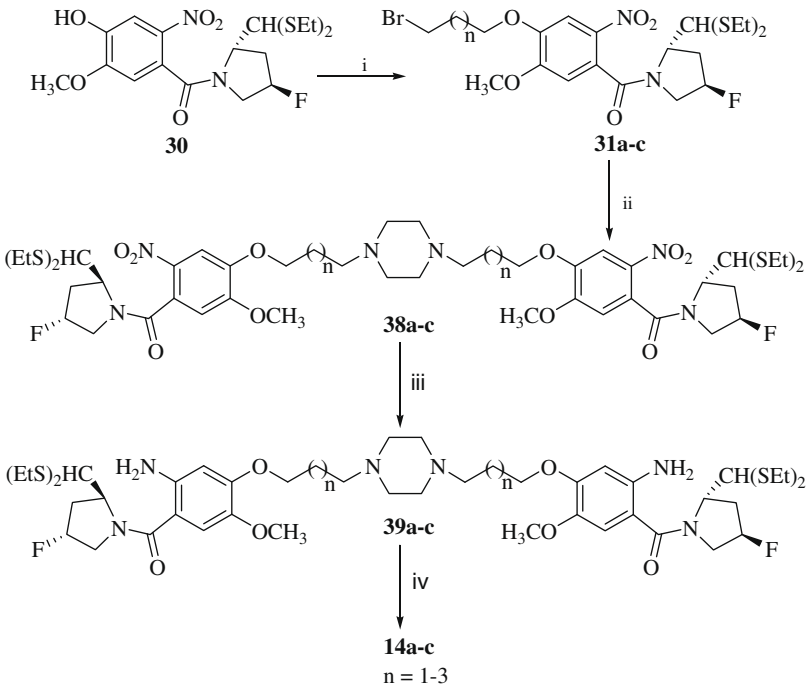
2.2. Biological activity

2.2.1. DNA interaction: thermal denaturation studies

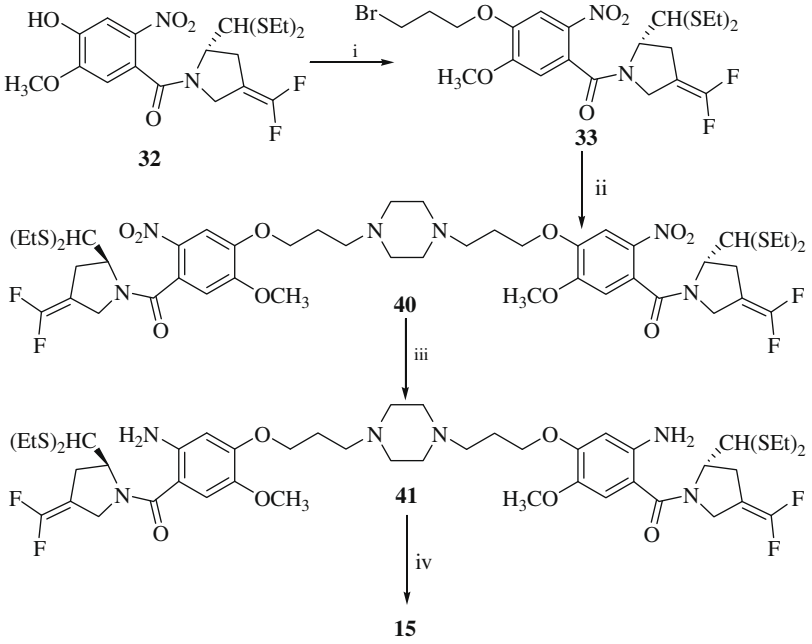
DNA-binding ability of these C2-fluoro substituted dimers **8a–c** and dimers with incorporation of piperazine in their linker spacer like **13a–c**, **14a–c** and **15** have been investigated by thermal denaturation studies using calf thymus (CT) DNA. Melting studies show that these compounds stabilize the thermal helix coil or melting



Scheme 4. Reagents and conditions: (i) dibromoalkanes, K_2CO_3, acetone, reflux, 48 h; (ii) piperazine, K_2CO_3, dry acetonitril, reflux, 48 h. (iii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux; 2 h; (iii) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O (4:1) or $Bi(OTf)_3{\cdot}xH_2O$, CH_2Cl_2–H_2O, 3–8 h.



Scheme 5. Reagents and conditions: (i) dibromoalkanes, K_2CO_3, acetone, reflux, 48 h; (ii) piperazine, K_2CO_3, dry acetonitril, reflux, 48 h; (iii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux; 2 h; (iii) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O (4:1) or $Bi(OTf)_3{\cdot}xH_2O$, CH_2Cl_2–H_2O, 3–8 h.



Scheme 6. Reagents and conditions: (i) 1,3-dibromopropane, K_2CO_3, acetone, reflux, 48 h; (ii) piperazine, K_2CO_3, dry acetonitril, reflux, 48 h; (iii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux; 2 h; (iii) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O (4:1) or $Bi(OTf)_3{\cdot}xH_2O$, CH_2Cl_2–H_2O, 3–8 h.

stabilization for the CT-DNA duplex at pH 7.0, and incubated at 37 °C with DNA/ligand molar ratio of 5:1. The increase in the helix melting temperature (ΔT_m) for each compound has been examined at 0 h. It is observed that these C2-fluoro substituted DC-81 dimers (**13a–c**, **14a–c**) enhance the helix melting temperature significantly in comparison to DSB-120 as shown in Table 1. Further, the compounds **8a** and **8b** also elevate the helix melting temperature considerably in comparison to DSB-120. Moreover, it is interesting to note that the incorporation of a piperazine moiety in the linker spacer in both the C2-monofluoro as well as difluoro substituted SJG-136 PBD dimers (**14a–c**, **15**) have exhibited remarkable DNA-binding affinity with ΔT_m values ranging from 28.9 to 38 °C. This enhancement in DNA-binding ability in comparison with non-fluoro PBD molecules is probably due to a more favorable fit of these molecules in the DNA minor groove. However, other factors such as strong non-covalent interactions and electro negativity aspect cannot be ruled out as observed in the previous studies in this laboratory. Moreover, the introduction of a pipera-

Table 1
Thermal denaturation data for C2-fluoro substituted pyrrolo[2,1-*c*][1,4] benzodiazepine dimers with calf thymus (CT) DNA

PBD Dimers	ΔT_m[a] (°C)
8a	13.1
8b	4.2
8c	18.9
13a	11.0
13b	14.0
13c	13.0
14a	37.0
14b	38.0
14c	37.0
15	28.9
DSB-120	10.2
SJG-136	25.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.1 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ±0.1 − 0.2 °C. For a 1:5 molar ratio of [PBD]/[DNA], where in CT-DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01].

zine moiety in the alkane spacer linker and group like fluoro substituent increases the hydrophobic interaction with DNA making them as more stable adducts. These results indicate the significance of fluoro substituent as well as the piperazine moiety towards the enhancement of the DNA-binding ability in the PBD based molecules. Interestingly mono fluoro substitution with the incorporation of a piperazine moiety in this spacer linker produced remarkable enhancement of the DNA-binding ability.

2.2.2. RED_{100}-restriction endonuclease digestion assay

Restriction endonuclease inhibition has been employed as a tool to confirm the relative binding affinity of DNA-interactive small molecule ligands.[35,36] A quantitative restriction enzyme digestion RED_{100} assay has been developed in which the inhibition of DNA cleavage by *Bam*H1 is used to probe the DNA-binding capability of PBD monomers.[37] Earlier we have investigated this assay for preferences of base pair selectivity of the imine-amide PBD-dimers.[19] Recently, this study has been carried out to determine the DNA-binding ability of benzothiadiazine–PBDs and phosphonate linked–PBD conjugates.[38,39] Moreover, this technique has also been used to study the covalent DNA interaction of the PBD dimers and is capable of distinguishing the monomeric and dimeric families.[40] Preincubation of PBD with DNA leads to the formation of DNA–drug complex, wherein the *Bam*H1 cleavage sequence G[1]GATCC overlaps with several favoured PBD binding sites suggesting that the ligand binding significantly inhibits the *Bam*H1 cleavage activity. The results of this experiment for representative compounds **8a**, **13a**, **14a** and **15** have been shown in Figure 2 and indicate that the PBD inhibits *Bam*H1 digestion in a dose-dependent manner with a stoichiometric ratio. It is observed that C2-fluoro substituted piperazine linked dimer **14a** significantly inhibits *Bam*H1. However, difluoro substituted piperazine linked dimer **13a** and **15** did not exhibit inhibitory action on *Bam*H1 compared to monofluoro substituted piperazine linked dimer **14a** and difluoro substitution of a non-piperazine linked dimer **8a**. Overall, compound **14a** shows promising inhibition restriction endonuclease *Bam*H1 and these results are in agreement with the DNA-binding affinity as determined by the thermal denaturation studies except for compounds **13a** and **15**.

2.2.3. Molecular modeling studies

The variations in the DNA-binding ability with respect to fluoro substitution in this class of dimers has been investigated by molecular modeling approaches employing docking studies. About 8 different DNA sequences have been examined to investigate the most suitable DNA sequence for taking up such docking studies. Among these, sequence A (5′-CGCAGAAATTTGTGCG-3′) has shown good comparison with the experimental results as this has optimal A-T rich region.

The GOLD 3.2 program protocol was used for the molecular docking calculations of the cross-linked complex formed between the synthesized PBD dimers and the host DNA duplex (5′-CGCA GAAATTTGTGCG-3′). The Gasteiger–Huckel charges were applied on the inhibitor and energy minimized using SYBY l6.9 (Tripos Inc., St. Louis, MO). The B-DNA duplex was built and minimized using NUCGEN and SANDER program of AMBER 8, respectively. The entire conformational space of the DNA molecule was scanned during docking. The parameter set for docking was as follows: number of islands 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1, and the van der Waals and hydrogen bonding were set to 4.0 and 2.5, respectively.

Based on the scores and intermolecular interactions such as Coulomb and van der Waals, this method was used to determine the sites in a biomolecule where the ligand would prefer to bind energetically. Molecular docking simulations were used to investigate the probable sites of PBD dimers interaction with the DNA, that is, whether major or minor grooves or specific sequences of nucleobases. It was found that the minor groove of the DNA structure is preferred over the major groove and sequence composed mainly of adenine and thymine bases are probable binding sites. Binding of the PBD ligand **14b** in the minor groove does not have a large impact on the conformation of the DNA, as enunciated by

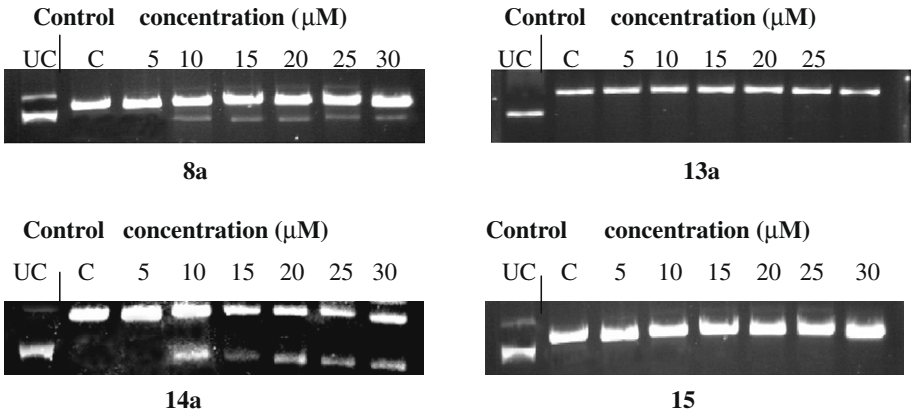


Figure 2. RED100-restriction endonuclease digestion assay for fluoro dimers with CT-DNA inhibitory activity of **8a**, **13a**, **14a** and **15** on the cleavage of plasmid pBR322 by restriction endonuclease *Bam*H1 (10 units in 1 μL) for 1 h at 37 °C. The cut (C) and uncut (UC) products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination. Lane 1: control pBR322; lane 2: complete digest of pBR322 by *Bam*H1.

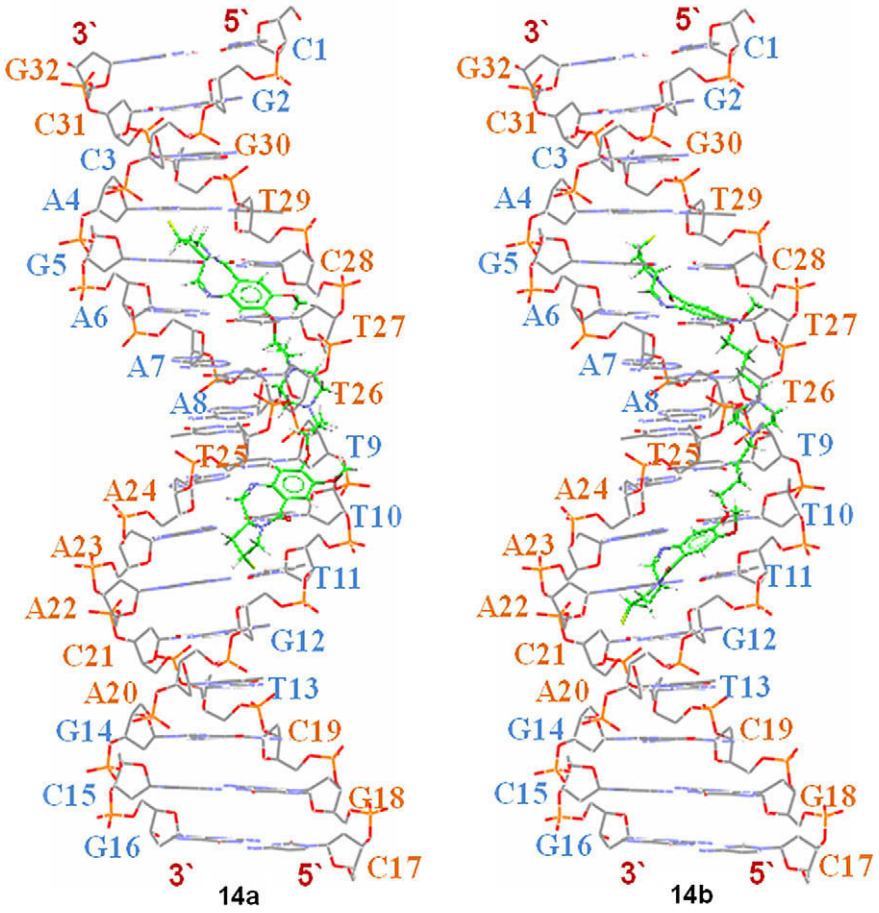


Figure 3. Docked structures of the PBD dimers **14a** and **14b** with DNA duplex (5′-CGCAGAAATTTGTGCG-3′).

the qualitative agreement between the RMSD of free DNA and the complex. The variation in the binding affinities **14a** and **14b** (gold score = 84.20 and 87.64, respectively) to DNA may be traced to the linker length differences in the PBD dimers and the electrostatic interactions due to the fluorine atom in the ligand, showing a preferred binding to AT rich regions in the sequence. The linker of PBD dimers localized in the six AT sites (5′-CGCAGAAATTTGTGCG-3′) are observed in each DNA-PBD complex. PBD appears to interact favorably with the electrostatic landscape of DNA (Fig. 3).

The fluorine atoms flanking the two ends of PBD dimer appear to interact with the thymine in **14a** at a distance of 2.544 Å (T11:O4′) and 2.290 Å (T11:O2) whereas in **14b** at a distance of 2.834 Å (T29:O3′) and 3.076 Å (T11:04′). One of the imine nitrogen (**10a**) of the PBD dimer **14a** interacts at a distance of 2.464 Å (A7:O4′) and of **14b** interacts with a distance of 2.329 Å (A7:O4′) and 2.651 Å (A6:3N). The other imine nitrogen (**10b**) of the PBD dimer **14a** interacts with the adenine ring of DNA at a distance of 2.652 Å (A24:3N), 2.378 Å (A24:O4′) and of **14b** interacts at a distance of 3.041 Å (A24:04′), 2.337 Å (A23:3N). This partitioning of anionic charge by PBD into the minor groove of AT base pairs may be a general feature of sequence-specific DNA–small molecule interactions and a potentially useful important factor in ligand design. The excellent agreement with the experimental observations validates the docking approaches used in this study. Thus the molecular modeling studies unambiguously identify and validate the binding mode of these PBD dimers with DNA.

2.2.4. Cytotoxicity

The representative compounds **8a**, **13a** and **14a** have been evaluated for in vitro anticancer activity in the standard 60 human cancer cell lines derived from nine cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancers). For each compound, dose–response curves for each cell line have been measured at a minimum of five concentrations at 10-fold dilutions. A protocol of 48 h continuous drug exposure is used, and a sulforhodamine B (SRB) protein assay[19] has been used to estimate cell viability or growth. The concentration causing 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI, 0% growth), and 50% cell death (LC_{50}, 50% growth) as compared with the control has been calculated. The GI_{50} values of these compounds (**8a**, **13a** and **14a**) have shown significant cytotoxic potency in the large number of cell lines with GI_{50} values in micromolar to nanomolar range. It is interesting to observe that compound **14a** exhibits strong effect against most of the cancer cell lines with $GI_{50} < 10^{-8}$ M (10 nM), similarly compounds **8a** also exhibits significant activity against respective cell lines with $GI_{50} < 10^{-8}$ M (10 nM). Compared to these two compounds **13a** shows moderate cytotoxic activity with $GI_{50} < 10^{-7}$ M (10 μM) as seen from data given in Table 2. Therefore, these results indicate that incorporation of a piperazine moiety in the alkane chain linker spacer is responsible for the enhancement of cytotoxic activity apart from the DNA-binding ability. Similarly, it is also observed that C2-fluoro substitution in these PBD dimers also enhances the anticancer activity compared to non-fluoro PBD dimers. The mean graph midpoint (MG MID) values of log TGI and log LC_{50} as well as log GI_{50} for **8a**, **13a** and **14a** are listed in Table 3. As demonstrated by mean graph pattern, these compounds exhibit an interesting profile of activity and selectivity for various cell lines. The MG MID of log TGI and log LC_{50} showed similar pattern to the log GI_{50} MG MID, therefore the in vitro cytotoxicity exhibited by these compounds is highly significant (Table 4).

Table 2
Docking fitness scores of the DNA–ligand interaction with different DNA sequences

Compounds	A	B	C	D	E	F	G	H
8a	67.00	67.21	70.40	70.84	70.41	71.84	66.18	62.22
8b	72.64	73.16	76.48	76.36	75.50	77.36	76.34	66.22
8c	81.63	79.30	84.99	80.28	84.76	82.28	76.92	72.28
13a	77.56	81.14	81.31	81.28	83.09	81.08	83.60	74.02
13b	81.47	88.46	81.71	80.48	81.28	80.38	86.88	84.11
13c	82.39	80.01	86.32	81.19	84.56	78.09	82.82	73.81
14a	84.20	76.42	79.47	77.10	81.05	77.39	77.58	72.58
14b	87.64	78.97	81.32	82.30	84.13	82.80	83.48	75.49
14c	79.69	77.52	89.84	85.48	80.49	85.82	78.77	80.50
15	79.88	75.20	73.53	78.65	78.15	77.65	73.96	77.37

A = 5′-CGCAGAAATTTGTGCG-3′.
B = 5′-CGCAGAAATTTCTGCG-3′.
C = 5′-CGCAGAAAATTTCTGCG-3′.
D = 5′-CGCAGAAAATTTTCTGCG-3′.
E = 5′-CGCAGAAAATTTGTGCG-3′.
F = 5′-CGCAGAAAATTTTGTGCG-3′.
G = 5′-AGCTTATAATGG-3′.
H = 5′-GGGGAGAGAGAGGGG-3′.

3. Conclusion

In this investigation a series of C2 fluorinated DC-81 and its dimers have been synthesized that exhibited significant DNA-binding ability with potential anticancer activity. Some representative compounds (**8a**, **13a** and **14a**) have been screened for their activity against various human cancer cell lines. Most of them possess promising anticancer activity having GI_{50} values in micromolar to nanomolar concentration range. These cytotoxicity results are useful in the further design of improved interstand DNA cross-linking molecules based on the PBD ring system. The restriction endonuclease studies also demonstrate the enhancement in their DNA-binding ability. This has been further validated by molecular modeling studies and showing a preferred binding to AT rich region in the sequences examined. Overall, these results suggest that the C2-fluoro substituted PBDs are biologically more potent than their C2-unsubstituted PBDs.

4. Experimental

Reaction progress was monitored by thin-layer chromatography (TLC) using GF254 silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 and 60–120 mesh). The majority of reaction solvents were purified by distillation under nitrogen from the indicated drying agents and used fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenoneketyl), methanol (magnesium methoxide), and acetonitrile (calcium hydride). ^1H NMR spectra were recorded on Varian Gemini 200 MHz spectrometer using tetra methyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) down field from tetra methyl silane. Spin multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Coupling constants are reported in Hertz (Hz). EI mass spectra were recorded on a VG-7070H Micro mass spectrometer at 200 °C, 70 eV, with a trap current of 200 1A and 4 kV of acceleration voltage. ESI spectra were recorded on Micro mass, Quattro LC using ESI^+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector.

5. General procedure

5.1. Methyl-(2*S*)-*N*-*tert*-butoxycarbonyl-4-hydroxyproline (18)

A mixture of *trans*-4-hydroxyproline methyl ester **17** (25 g, 0.19 mol) in 380 mL of a 2:1 mixture of THF/H_2O was treated first

Table 3
In vitro anticancer activity of compounds **8a**, **13a** and **14a** in selected human cancer cell lines

Cancer panel/cell line	GI_{50} (μM) **8a**	GI_{50} (μM) **13a**	GI_{50} (μM) **14a**
Leukemia			
CCRF-CEM	0.41	0.97	<0.01
HL-60(TB)	0.25	1.18	<0.01
K-562	0.53	1.74	<0.01
MOLT-4	0.08	2.02	<0.01
RPMI-8226	1.67	2.20	<0.02
SR	<0.06	0.88	<0.01
Non-small cell lung			
A549/ATCC	1.21	7.32	0.12
EKVX	1.72	—	0.17
HOP-62	0.10	3.17	<0.01
HOP-92	—	6.14	<0.09
NCI-H226	0.62	—	0.10
NCI-H23	0.49	4.66	<0.03
NCI-H322M	2.19	—	0.26
NCI-H460	<0.08	2.60	<0.01
NCI-H522	0.14	1.14	<0.01
Colon			
COLO 205	0.58	3.09	<0.04
HCC-2998	1.03	5.40	0.11
HCT-116	0.13	3.59	<0.02
HCT15	8.62	—	2.56
HT-29	0.27	3.61	0.17
KM12	2.07	4.10	0.10
SW-620	0.31	2.61	<0.05
CNS			
SF-295	<0.03	—	—
SF-268	—	3.57	<0.01
SF-539	0.15	3.05	<0.01
SNB-19	0.35	4.08	<0.02
SNB-75	0.08	5.52	<0.03
U251	0.11	3.36	<0.01
Melanoma			
LOX IMVI	0.20	1.89	<0.01
MALME-3M	1.03	2.80	<0.02
M14	—	3.25	<0.03
SK-MEL-2	1.62	—	0.50
SK-MEL-5	1.13	1.86	<0.03
SK-MEL-28	—	4.28	<0.04
UACC-257	1.33	—	1.27
UACC-62	0.41	3.05	<0.08
Ovarian			
IGROV1	0.11	1.92	<0.05
OVCAR-3	0.94	3.55	<0.04
OVCAR-4	1.45	—	0.46
OVCAR-5	0.75	3.83	0.16
OVCAR-8	0.42	6.30	0.10
SK-OV-3	0.60	8.64	<0.07
Renal			
786-0	<0.01	3.17	<0.03
A498	0.61	2.43	<0.06
ACHN	0.65	5.64	0.15
CAKI-1	<0.01	3.31	0.02
RXF 393	—	2.14	0.11
SN12C	0.17	4.05	<0.01
TK-10	3.45	4.38	0.25
UO-31	1.05	—	1.03
Prostate			
PC-3	<0.08	3.06	0.01
DU-145	0.37	3.63	0.04
Breast			
MCF7	0.13	2.02	<0.01
NCI/ADR-RES	—	—	5.25
MDA-MB-231/ATCC	0.80	4.29	0.13
HS 578T	0.17	2.32	<0.04
MDA-MB-435	0.57	2.94	<0.03
BT-549	0.41	1.95	<0.09
T-47D	0.24	3.25	<0.01
MDA-MB-468	—	1.38	<0.01

Table 4
$\log_{10}$ GI_{50} $\log_{10}$ TGI and $\log_{10}$ LC_{50} mean graphs midpoint (MG_MID) of in vitro cytotoxicity data for the compounds **8a**, **13a** and **14a** against human tumor cell lines

Compound	$\log_{10}$ GI_{50}	$\log_{10}$ TGI	$\log_{10}$ LC_{50}
8a[a]	−6.38	−5.38	−4.40
13a[a]	−5.41	−4.79	−4.20
14a[a]	−7.28	−6.12	−4.65

[a] Compound **8a**, **13a** and **14a** has been selected and evaluated in the standard 60 cell line cancer screen of the National Cancer Institute (NCI), Maryland, USA.

with 10% aqueous NaOH (80 mL) and then with di-*tert*-butyldicarbonate (60 g, 0.28 mol). The reaction mixture was stirred at room temperature overnight and then THF was removed in vacuum. The residue was adjusted to pH 2 by the addition of 10% aqueous $KHSO_4$. The acidic solution was extracted several times with ethyl acetate. The combined organic extracts were the desiccant and evaporation of the solvent in vacuum provides the product **18** as syrup, which was used without further purification.

Yield 44 g, 100%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.38, 1.45 (2s, 9H), 1.95–2.10 (m, 1H), 2.20–2.38 (m, 1H), 3.38–3.62 (m, 2H), 3.75 (s, 3H), 4.25–4.48 (m, 2H). ESI MS *m/z* 246 $[M+1]^+$. Anal. Calcd for $C_{11}H_9NO_5$: C, 53.87; H, 7.81; N, 5.71. Found: C, 53.85; H, 7.60; N, 5.48.

5.2. Methyl-(2*S*)-*N*-*tert*-butoxycarbonyl-4-oxopyrrolidine (19)

Trichloroisocyanuric acid (1.87 g, 8.09 mmol) was added to a solution of the methyl-(2*S*)-*N*-*tert*-butoxycarbonyl-4-hydroxyproline (**18**) (2 g, 8.09 mmol) in dichloromethane (20 mL), and the solution was stirred and maintained at 0 °C, followed by the addition of TEMPO (0.012 g, 0.08 mmol). After the addition, the mixture was warmed to room temperature and stirred for 15 min and then filtered on Celitebed, and the organic phase was washed with saturated Na_2CO_3 (20 mL), followed by dilute HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated to afford the methyl-(2*S*)-*N*-*tert*-butoxycarbonyl-4-oxopyrrolidine (**19**) that was used without further purification.

Yield 1.86 g, 95%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.44–1.50 (2s, 9H), 2.54–2.64 (m, 1H), 2.91–2.97 (m, 1H), 3.75 (s, 3H), 4.08–4.18 (m, 2H), 4.40–4.60 (dd, J = 10.3, 9.3 Hz, 1H). ESI MS *m/z* 244 $[M+1]^+$. Anal. Calcd for $C_{11}H_{17}NO_5$: C, 54.31; H, 7.04; N, 5.76. Found: C, 54.25; H, 7.00; N, 5.58.

5.3. Methyl-(2*S*)-*N*-*tert*-butoxycarbonyl-4,4-difluoropyrrolidine (20)

To a solution of compound **19** (120 mg, 0.49 mmol) in dry dichloromethane (30 mL) cooled at −78 °C was added a solution of *N*,*N*-diethylaminosulfortrifluoride (DAST) (0.16 mL, 1.22 mmol) in dry dichloromethane (15 mL) over a period of 30 min. This reaction mixture was continuously stirred for 12 h at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was washed with ice-water (20 mL), the aqueous layer was extracted with dichloromethane. The extract was neutralized with 5% sodium bicarbonate solution and dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum to afford the crude product. This was further purified by column chromatography (20% EtOAc–hexane) to afford the compound **20**.

Yield 105 mg, 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45–1.52 (2s, 9H), 2.60–2.75 (m, 1H), 2.85–3.00 (m, 1H), 3.75 (s, 3H), 4.08–4.18 (m, 2H), 4.40–4.60 (dd, J = 9.2, 9.2 Hz, 1H). ESI MS *m/z* 266 $[M+1]^+$. Anal. Calcd for $C_{11}H_{17}NO_4$: C, 49.81; H, 6.46; N, 5.28. Found: C, 49.52; H, 6.24; N, 5.09.

5.4. Methyl-(2*S*)-4,4-difluoropyrrolidine-2-carboxylate (21)

To a solution of Boc-protected compound **20** (3.0 g, 11.24 mmol) in dry dichloromethane was added trifluoroacetic acid (8.63 mL, 112.37 mmol) at 0 °C and stirred under nitrogen for 8 h, the reaction mixture was then concentrated in vacuum to afford the compound **21** and then it was used directly in the next step.

5.5. Methyl-(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxylate (23)

A catalytic amount of DMF (2 drops) was added to a stirred solution of 4-(benzyloxy)-5-methoxy-2-nitrobenzoic acid **22** (5.11 g, 16.86 mmol) and thionyl chloride (1.82 mL, 25.25 mmol) in dry benzene (100 mL) and the mixture was stirred for overnight under nitrogen atmosphere. The benzene was evaporated under vacuum and the resulting acid chloride was dissolved in dry THF (40 mL) and added drop wise over a period of 30 min to a stirred suspension of methyl-(2*S*)-4,4-difluoropyrrolidine-2-carboxylate (**21**) (3 g, 18.18 mmol), triethylamine (3.94 mL, 54.55 mmol) and ice water (2 mL) in dry THF cooled in an ice bath under nitrogen atmosphere. After the addition was completed, the reaction mixture was brought to ambient temperature and stirred for an additional hour. The THF was evaporated under vacuum and the aqueous layer was washed with ethyl acetate (25 mL). The aqueous phase was then adjusted to pH 3 using 6 N HCl and extracted with ethyl acetate and washed with brine. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (50% EtOAc–hexane) to afford the compound **23** as a white solid.

Yield 4.62 g, 70%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.40–3.10 (m, 2H), 3.40–4.45 (m, 8H), 4.95 (d, J = 4.2 Hz, 1H), 5.20 (s, 2H), 6.85 (s, 1H), 7.40–7.60 (m, 5H) 7.75 (s, 1H) ESI MS *m/z* 450 $[M]^+$. Anal. Calcd for $C_{21}H_{20}F_2N_2O_7$: C, 56.00; H, 4.48; N, 6.22. Found: C, 55.91; H, 4.32; N, 6.14.

5.6. (2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (25)

Diisobutylaluminium hydride (DIBAL-H) solution (14.44 mL of 1 M solution in toluene, 2.02 equiv) was added drop wise over a period of 30 min to a vigorously stirred solution of methyl-(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxylate (**23**) (3.0 g, 6.90 mmol) in anhydrous dichloromethane (25 mL) under nitrogen at −78 °C (dry ice/acetone bath). After the mixture was stirred for an additional 30 min excess reagent was decomposed by careful addition of methanol (10 mL) followed by 5% HCl (15 mL). The resulting mixture was allowed to warm to room temperature and the organic layer was removed. The aqueous layer, washed with brine solution (2 × 40 mL), dried over anhydrous $NaSO_4$ and the solvent was evaporated under vacuum, to afford the crude aldehyde, which was purified by column chromatography (55% EtOAc–hexane) to afford the pure compound (2S)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde which was dissolved in dry chloroform (40 mL) the ethanethiol (1.18 mL, 15.93 mmol) was added to a stirred solution of the (2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde (3.0 g, 7.23 mmol) in dry chloroform (40 mL) under nitrogen atmosphere. The mixture was stirred for a further 30 min followed by the addition of trimethylsilyl chloride (2.30 mL, 18.04 mmol) and stirring was continued for further 16–18 h. The progress of the reaction mixture was carefully neutralized with sodium bicarbonate solution and then extracted with chloroform (2 × 50 mL). The combined organic layers dried over anhydrous Na_2SO_4 and evaporated under vacuum to

get the crude product. This was purified by column chromatography (40% EtOAc–hexane) to afford the compound **25** as white solid.

Yield 3.0 g, 79%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.20–1.45 (m, 6H), 2.28–2.95 (m, 6H), 3.30–4.30 (m, 5H), 4.58 (d, J = 4.12 Hz, 1H), 5.08 (m, 1H), 5.20 (s, 2H), 6.74 (s, 1H), 7.30–7.60 (m, 5H) 7.73 (s, 1H) ESI MS m/z 528 $[M+2]^+$. Anal. Calcd for $C_{24}H_{28}F_2N_2O_5S_2$: C, 54.74; H, 5.36; N, 5.32. Found: C, 54.60; H,5.25; N, 5.15.

5.7. (2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-aminobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (28)

The compound **25** (260 mg, 0.59 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 2.99 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **28** as yellow liquid (210 mg, 88%). Which due to potential stability problems directly used in the next step.

5.8. (11a*S*)-2,2-Difluoro-8-benzyloxy-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*] [1,4]benzodiazepin-5-one (4)

A solution of **28** (616 mg, 1 mmol), $HgCl_2$ (677 mg, 2.5 mmol) and $CaCO_3$ (250 mg, 2.5 mmol) in CH_3CN–H_2O (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a Celitebed. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL) the organic layer was washed with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried on ($NaSO_4$). The organic layer was evaporated in vacuum and purified by column chromatography (95% $CHCl_3$–CH_3OH) to give compound **4**. This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

Yield 295 mg, 60%; $[\alpha]_{26}^{D}$ −24.0 (c 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.55–3.00 (m, 2H), 3.38–4.00 (m, 4H), 4.60–4.80 (m, 1H), 5.18 (s, 2H), 6.24 (s, 1H), 6.70 (s, 1H), 7.30–7.55 (m, 5H), 7.78 (d, J = 4.2 Hz, 1H). ESI MS m/z 372 $[M]^+$. Anal. Calcd for $C_{20}H_{18}F_2N_2O_3$: C, 64.51; H, 4.87; N, 7.52. Found: C, 64.22; H, 4.66; N, 7.24.

5.9. (2*S*)-*N*-(4-Hydroxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (26)

To a stirred solution of EtSH (5.35 mL, 72.27 mmol) and $BF_3{\cdot}OEt_2$ (4.74 mL, 38.01 mmol) was added a solution of compound **25** (2.0 g, 3.80 mmol) in dichloromethane (50 mL) at room temperature. Stirring was continued until the reaction was completed as indicated by TLC. It was diluted with dichloromethane and quenched with saturated $NaHCO_3$ (20 mL). The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic layer were washed with brine (1 × 25 mL), dried over anhydrous Na_2SO_4 and the solvent removed in vacuum to afford the crude product. This was further purified by column chromatography (50% EtOAc–hexane) to get pure compound **26** as white solid.

Yield 1.3 g, 78%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35–1.45 (m, 6H), 2.50–2.95 (m, 6H), 3.48–3.87 (m, 2H), 4.00 (s, 3H), 4.80 (d, J = 4.20 Hz, 1H), 4.88–4.98 (m, 1H), 6.60 (s, 1H), 6.75 (s, 1H), 7.65 (s, 1H) ESI MS m/z 437.6 $[M+1]^+$. Anal. Calcd for $C_{17}H_{22}F_2N_2O_5S_2$: C, 46.78; H, 5.08; N, 6.42. Found C, 46.40; H, 4.99; N, 6.25.

5.10. (2*S*)-*N*-(4-Hydroxy-5-methoxy-2-aminobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (27)

The compound **26** (260 mg, 0.59 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 2.99 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **27** as yellow liquid (210 mg, 88%), which was directly used in the next step due to potential stability problems.

5.11. (11a*S*)-2,2-Difluoro-8-hydroxy-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*] [1,4]benzodiazepin-5-one (5)

A solution of compound **27** (616 mg, 1 mmol), $HgCl_2$ (677 mg, 2.5 mmol) and $CaCO_3$ (250 mg, 2.5 mmol) in CH_3CN–H_2O (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a Celitebed. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL) the organic layer was washed with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried on ($NaSO_4$). The organic layer was evaporated in vacuum and purified by column chromatography (95% $CHCl_3$–CH_3OH) to give compound **5** this material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

Yield 85 mg, 55%; $[\alpha]_{D}^{26}$ +4.0 (c 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.60–3.10 (m, 2H), 3.80–4.28 (m, 5H), 4.60 (m, 1H), 6.57 (s, 1H), 6.84 (s, 1H), 7.50 (s, 1H), 7.84 (d, J = 4.3 Hz, 1H), ESI MS m/z 282 $[M]^+$. Anal. Calcd for $C_{13}H_{12}F_2N_2O_3$: C, 55.32; H, 4.29; N, 9.93. Found: C, 55.21; H, 4.18; N, 9.79.

5.12. General procedure for the synthesis of compounds (34a–c)

5.12.1. 1,1′-{[(Propane-1,3-diyl)dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoropyrrolidine-2-carboxaldehydediethylthioacetal] (34a)

To a solution of (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (300 mg, 0.69 mmol) in dry acetone (30 mL) was added anhydrous K_2CO_3 (381 mg, 2.76 mmol) and the 1,3-dibromopropane (0.035 mL, 0.34 mmol). The reaction mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC. After completion of the reaction potassium carbonate was removed by filtration and the filtrate was evaporated under vacuum to get the crude product. The crude product was purified by column chromatography (60% EtOAc–hexane) to afford the compound **34a** as yellow solid.

Yield 420 g, 67%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.25–1.40 (m, 12H), 2.15–2.50 (m, 6H), 2.60–2.95 (m, 8H), 3.40–3.85 (m, 4H), 3.95 (s, 6H), 4.25–4.45 (t, J = 5.6 Hz, 4H), 4.75 (d, J = 4.0 Hz, 2H), 4.82–4.95 (m, 2H), 6.75 (s, 2H), 7.70 (s, 2H) FAB MS m/z 914 $[M+1]^+$. Anal. Calcd for $C_{37}H_{48}F_4N_4O_{10}S_4$: C, 48.67; H, 5.30; N, 6.14. Found: C, 48.51; H, 5.19; N, 6.00.

5.12.2. 1,1′-{[(Butane-1,4-diyl)dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoropyrrolidine-2-carboxaldehydediethylthioacetal] (34b)

The compound **34b** was prepared following the method described for the compound **34a**, employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (300 mg, 0.69 mmol), 1,4-dibromobutane (0.041 mL, 0.34 mmol) and K_2CO_3 (381 mg, 2.76 mmol) in dry acetone, the crude product was purified by column chromatography (60% EtOAc–hexane) to afford the compound **34b** as white solid.

Yield 410 g, 64%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.29–1.40 (m, 12H), 2.05–2.20 (m, 4H), 2.40–3.00 (m, 12H), 3.40–3.85 (m, 4H),

3.90 (s, 6H), 4.25 (t, J = 5.4 Hz, 4H), 4.75 (m, 2H), 4.83–4.95 (m, 2H), 6.70 (s, 2H), 7.65 (s, 2H) ESI MS m/z 928 $[M+1]^+$. Anal. Calcd for $C_{38}H_{50}F_4N_4O_{10}S_4$: C, 49.23; H, 5.44; N, 6.04. Found: C, 49.02; H, 5.20; N, 5.79.

5.12.3. 1,1′-{[(Pentane-1,5-diyl)dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoropyrrolidine-2-carboxaldehydediethylthioacetal] (34c)

The compound **34c** was prepared following the method described for the compound **34a**, employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzyol)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (300 mg, 0.69 mmol), 1,5-dibromopentane (0.041 mL, 0.34 mmol) and K_2CO_3 (381 mg, 2.76 mmol) in dry acetone, the crude product was purified by column chromatography (60% EtOAc–hexane) to afford the compound **34c** as white solid.

Yield 440 g, 70%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.35–1.45 (m, 12H), 1.75–1.85 (m, 6H), 1.95–2.95 (m, 12H), 3.50–3.60 (m, 4H), 4.05 (s, 6H), 4.20 (m, 4H), 4.85–4.95 (m, 4H), 6.80 (s, 2H), 7.75 (s, 2H), FAB MS m/z 928 $[M+1]^+$. Anal. Calcd for $C_{39}H_{52}F_4N_4O_{10}S_4$: C, 49.77; H, 5.57; N, 5.95. Found: C, 49.62; H, 5.45; N, 5.82.

5.13. General procedure for the synthesis of compounds (35a–c)

5.13.1. 1,1′-{[(Propane-1,3-diyl)dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoro-pyrrolidine-2-carboxaldehydediethylthioacetal] (35a)

The compound **34a** (210 mg, 0.23 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (518 mg, 2.30 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 25 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **35a** as yellow liquid (180 mg, 92%), which was directly used in the next step due to potential stability problems.

5.13.2. 1,1′-{[(Butane-1,4-diyl)dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoropyrrolidine-2-carboxaldehydediethylthioacetal] (35b)

The compound **35b** was prepared following the method described for the compound **35a**, employing the compound **34b** (410 mg, 0.44 mmol) to afford the amino diethyl thioacetal **35b** as a yellow oil (360 mg, 94%).

5.13.3. 1,1′-{[(Pentane-1,5-diyl)dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]}-bis[4,4-difluoropyrr-olidine-2-carboxaldehydediethylthioacetal] (35c)

The compound **35c** was prepared following the method described for the compound **35a**, employing the compound **34c** (330 mg, 0.35 mmol) to afford the amino diethyl thioacetal **35c** as a yellow oil (280 mg, 91%).

5.14. General procedure for the synthesis of compounds (8a–c)

5.14.1. 1,1′-[Propane-1,3-diyl)dioxy]-bis[(11a*S*)-7-methoxy-2, 2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one] (8a)

A solution of **35a** (180 mg, 0.21 mmol), $HgCl_2$ (341 mg, 1.26 mmol) and $CaCO_3$ (126 mg, 1.26 mmol) in CH_3CN–H_2O (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a Celitebed. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL) the organic layer was washed with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried on ($NaSO_4$). The organic layer was evaporated in vacuum and purified by column chromatography (60% ethyl acetate–hexane) to give compound **8a** this material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

Yield 295 mg, 60%; $[\alpha]_D^{26}$ +56.0 (c 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.85–2.05 (m, 2H), 2.20–2.65 (m, 4H), 3.52–3.85 (m, 6H), 3.94 (s, 6H), 4.00–4.25 (m, 4H), 6.80 (s, 2H), 7.44 (s, 2H), 7.79 (d, J = 5.0 Hz, 2H), FAB MS m/z 605 $[M+1]^+$. Anal. Calcd for $C_{29}H_{28}F_4N_4O_6$: C, 57.67; H, 4.67; N, 9.27. Found: C, 57.14; H, 4.40; N, 8.82.

5.14.2. 1,1′-[Butane-1,4-diyl)dioxy]-bis[(11a*S*)-7-methoxy-2, 2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one] (8b)

The compound **8b** was prepared following the method described for the compound **8a**, employing the compound **35b** (300 mg, 0.35 mmol) to afford the compound **8b** as a pale yellow solid.

Yield 123 mg, 55%; $[\alpha]_{26}^{D}$ +82.0 (c 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.85–2.15 (m, 4H), 2.25–2.85 (m, 4H), 3.50–3.83 (m, 6H), 3.93 (s, 6H), 4.01–4.35 (m, 4H), 6.80 (s, 2H), 7.43 (s, 2H), 7.78 (d, J = 5.2 Hz, 2H), ESI MS m/z 620 $[M+2]^+$. Anal. Calcd for $C_{30}H_{30}F_4N_4O_6$: C, 58.25; H, 4.89; N, 9.06; Found: C, 58.12; H, 4.65; N, 8.92.

5.14.3. 1,1′-[Pentane-1,5-diyl)dioxy]-bis[(11a*S*)-7-methoxy-2, 2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one] (8c)

The compound **8c** was prepared following the method described for the compound **8a**, employing the compound **35c** (330 mg, 0.35 mmol) to afford the compound **8c** as a pale yellow solid.

Yield 133 mg, 58%; $[\alpha]_{26}^{D}$ +50.0 (c 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.80–2.05 (m, 6H), 2.10–2.40 (m, 4H), 2.70–2.95 (m, 6H), 3.93(s, 6H), 3.99–4.25 (m, 4H), 6.80 (s, 2H), 7.45 (s, 2H), 7.79 (d, J = 5.1 Hz, 2H), FAB MS m/z 633 $[M+1]^+$. Anal. Calcd for $C_{31}H_{32}F_4N_4O_6$: C, 58.86; H, 5.10; N, 8.86. Found: C, 58.64; H, 5.00; N, 8.64.

5.15. General procedure for the synthesis of compounds (29a–c)

5.15.1. (2*S*)-*N*-[4-(4-Bromopropoxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (29a)

To a solution of (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (182 mg, 1 mmol) in acetone (30 mL) were added, anhydrous K_2CO_3 (553 mg, 4 mmol) and 1,3-dibromopropane (561 mg, 3 mmol) and the mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (10% EtOAc–hexane) to afford the compound **29a** as a white solid.

Yield 236 mg, 82%; ^{1}H NMR ($CDCl_3$): δ 1.36–1.42 (m, 6H), 2.39–2.47 (m, 2H), 2.62–2.95 (m, 6H), 3.48–3.58 (m, 2H), 3.64 (t, J = 6.0 Hz, 2H), 3.96 (s, 3H), 4.26 (t, J = 5.2 Hz, 2H), 4.82 (d, 1H), 4.89–4.96 (m, 1H), 6.77 (s, 1H), 7.72 (s, 1H) LC MS: m/z 580 $[M+Na]^+$. Anal. Calcd for $C_{20}H_{27}BrF_2N_2O_5S_2$: C, 43.09; H, 4.88; N, 5.03. Found: C, 42.95; H, 4.78; N, 4.95.

5.15.2. (2*S*)-*N*-[4-(5-Bromobutanoxy)-5-methoxy-2-nitrobenzoyl]-4,4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (29b)

The compound **29b** was prepared following the method described for the compound **29a**, by employing (2*S*)-*N*-(4-hydroxy-

5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (182 mg, 1 mmol) and 1,4-dibromopropane (605 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **29b** as white solid.

Yield 260 mg, 86%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.28–1.40 (m, 6H), 2.0–2.2 (m, 4H), 2.58–2.79 (m, 6H), 3.51 (t, 2H), 3.75 (m, 2H), 3.96 (s, 3H), 4.10 (t, 2H), 4.79 (d, 1H), 4.85 (m, 1H), 6.74 (s, 1H) 7.6 (s, 1H) LC MS: *m/z* 594 $[M+Na]^+$. Anal. Calcd for $C_{21}H_{29}BrF_2N_2O_5S_2$: C, 44.13; H, 5.11; N, 4.90. Found: C, 44.0; H, 5.0; N, 4.75.

5.15.3. (2*S*)-*N*-[4-(6-Bromopentanoxy)-5-methoxy-2-nitrobenzoyl]-4,4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (29c)

The compound **29c** was prepared following the method described for the compound **29a**, by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**26**) (182 mg, 1 mmol) and 1,5-dibromopentane (605 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **29c** as white solid.

Yield 255 mg, 85%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.06–1.36 (m, 6H), 1.40–2.1 (m, 4H), 2.35–2.49 (m, 2H), 2.58–2.88 (m, 6H), 3.52 (t, 2H), 3.70–3.97 (m, 2H), 3.97 (s, 3H), 4.15 (t, 2H), 4.80 (d, 1H), 4.91–5.02 (m, 1H), 6.75 (s, 1H), 7.6 (s, 1H) LC MS: *m/z* 608 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{31}BrF_2N_2O_5S_2$: C, 45.13; H, 5.34; N, 4.78. Found: C, 45.10; H, 5.20; N, 4.65.

5.16. General procedure for the synthesis of compounds (36a–c)

5.16.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitro-benzoyl-(4, 4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (36a)

To a solution of (2*S*)-*N*-[4-(4-bromopropoxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoro pyrrolidine-2-carboxaldehyde diethylthioacetal (**29a**) (507 mg, 1 mmol) in dry acetonitril (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the piperazine (43 mg, 0.5 mmol). The reaction mixture was refluxed for 48 h. The reaction was monitored by TLC using $CHCl_3$/MeOH (9:1) as a solvent system. The potassium carbonate was removed by suction filtration and the solvent was evaporated under vacuum. The crude product was purified by column chromatography (5% $CHCl_3$–MeOH) to afford the compound **36a** as a pale yellow solid.

Yield 845 mg, 90%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25–1.39 (m, 12H), 2.0–2.14 (m, 4H), 2.58–2.66 (m, 8H), 2.69–2.88 (m, 12H), 3.45–3.79 (m, 8H), 3.94 (s, 6H), 4.1 (t, 4H), 4.78 (d, 2H), 4.85–4.96 (m, 2H), 6.7 (s, 2H), 7.6 (s, 2H) ESI MS: *m/z* 1039 $[M]^+$. Anal. Calcd for $C_{44}H_{62}F_4N_6O_{10}S_4$: C, 50.85; H, 6.01; N, 8.09. Found: C, 50.85; H, 5.90; N, 7.95.

5.16.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitrobenzoyl-(4, 4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (36b)

The compound **36b** was prepared following the method described for the compound **36a**, employing piperazine and (2*S*)-*N*-[4-(5-bromobutanoxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**29b**) (521 mg, 1 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **36b** as pale yellow solid (860 mg, 85%).

Yield 860 mg, 85%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.33–1.48 (m, 12H), 1.66–1.98 (m, 8H), 2.40–2.50 (m, 8H), 2.63–2.94 (m, 12H), 3.42–3.83 (m, 8H), 3.92 (s, 6H), 4.11 (t, 4H), 4.77 (d, 2H), 4.83–4.94 (m, 2H), 6.72 (s, 2H), 7.62 (s, 2H) ESI MS: *m/z* 1067 $[M]^+$. Anal. Calcd for $C_{46}H_{66}F_4N_6O_{10}S_4$: C, 51.77; H, 6.23; N, 7.87. Found: C, 51.65; H, 6.00; N, 7.60.

5.16.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitro-benzoyl-(4, 4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (36c)

The compound **35c** was prepared following the method described for the compound **36a**, employing piperazine and (2*S*)-*N*-[4-(6-bromopentanoxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**29c**) (521 mg, 1 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **36b** as pale yellow solid.

Yield 850 mg, 75%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.34–1.40 (m, 12H), 1.47–1.96 (m, 12H), 2.36–2.49 (m, 4H), 2.50–2.66 (m, 8H), 2.68–2.90 (m, 8H), 3.37–3.80 (m, 8H), 3.94 (s, 6H), 4.08 (t, 4H), 4.77(d,2H), 4.85–4.91 (m, 2H), 6.72 (s, 2H), 7.63 (s, 2H) ESI MS: *m/z* 1095 $[M]^+$. Anal. Calcd for $C_{48}H_{70}F_4N_6O_{10}S_4$: C, 52.63; H, 6.44; N, 7.67. Found: C, 52.50; H, 6.30; N, 7.55.

5.17. General procedure for the synthesis of compounds (37a–c)

5.17.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydro-piperazine]-dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4,4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (37a)

The compound **36a** (260 mg, 0.59 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 2.99 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 25 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **37a** as yellow liquid (210 mg, 88%), which was directly used in the next step due to potential stability problems.

5.17.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4, 4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (37b)

The compound **37b** was prepared following the method described for the compound **37a**, employing the compound **36b** (967 mg, 1 mmol) to afford the amino diethylthioacetal **37b** as a yellow liquid (650 mg, 80%), which was directly used in the next step due to potential stability problems.

5.17.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4,4-difluoropyrrolidin-2-carboxaldehydediethylthioacetal)] (37c)

The compound **37c** was prepared following the method described for the compound **37a**, employing the compound **36c** (967 mg, 1 mmol) to afford the amino diethylthioacetal **37c** as a yellow liquid (685 mg, 83%), which was directly used in the next step due to potential stability problems.

5.18. General procedure for the synthesis of compounds (13a–c)

5.18.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydropip-erazine] dioxy}-bis[(11a*S*)-2,2-di-fluoro-7-methoxy-1,2,3,11a-tetrahydro--5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (13a)

A solution of amino thioacetal **37a** (878 mg, 1 mmol), $HgCl_2$ (1.19 g, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in CH_3CN/H_2O (4:1) was stirred slowly at room temperature overnight until TLC indicated the complete disappearance of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and

filtered through a Celite. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (95% $CHCl_3$–MeOH) to afford the compound **13a** as a pale yellow solid. This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine for.

Yield 526 mg, 65%; $[\alpha]_{26}^{D}$ +38.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.45–1.92 (m, 4H), 1.99–2.20 (m, 4H), 2.48–2.99 (m, 8H), 3.1–3.88 (m, 10H), 3.96 (s, 6H), 3.98–4.24 (m, 4H), 6.80 (s, 2H), 7.49 (s, 2H), 7.82 (d, *J* = 3.8 Hz, 2H). ESI MS: *m/z* 731 $[M+1]^+$. Anal. Calcd for $C_{36}H_{42}F_4N_6O_6$: C, 59.17; H, 5.79; N, 11.50. Found: C, 59.10; H, 5.60; N, 11.40.

5.18.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydropiper-azine] dioxy}-bis[(11a*S*)-2,2-di-fluoro-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (13b)

The compound **13b** was prepared following the method described for the compound **13a**, employing the compound **37b** (955 mg, 1 mmol) to afford the compound **13b** as a pale yellow solid.

Yield 599 mg, 60%; $[\alpha]_{26}^{D}$ +52.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.56–1.94 (m, 8H), 1.99–2.35 (m, 4H), 2.49–2.99 (m, 8H), 3.29–3.87 (m, 10H), 3.93 (s, 6H), 3.98–4.37 (m, 4H), 6.89 (s, 2H), 7.46 (s, 2H), 7.83 (d, *J* = 3.67 Hz, 2H). Anal. Calcd for $C_{38}H_{46}F_4N_6O_6$: C, 60.15; H, 6.11; N, 11.08. Found: C, 59.95; H, 6.05; N, 11.0.

5.18.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-2,2-di-fluoro-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (13c)

The compound **13c** was prepared following the method described for the compound **13a**, employing the compound **37c** (967 mg, 1 mmol) to afford the compound **13c** as a pale yellow solid.

Yield 618mg, 64%; $[\alpha]_{26}^{D}$ +51.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.54–2.03 (m, 12H), 2.22–2.47 (m, 4H), 2.50–2.73 (m, 8H), 3.35–3.85 (m, 10H), 3.94 (s, 6H), 3.97–4.26 (m, 4H), 6.71 (s, 2H), 7.47 (s, 2H), 7.79 (d, *J* = 3.6, 2H) ESI MS: *m/z* 787 $[M+1]^+$. Anal. Calcd for $C_{40}H_{50}F_4N_6O_6$: C, 61.06; H, 6.40; N, 10.68. Found: C, 60.89; H, 6.25; N, 10.57.

5.19. General procedure for the synthesis of compounds (31a–c)

5.19.1. (2*S*)-*N*-[4-(4-Bromopropoxy)-5-methoxy-2-nitro-benzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diet-hylthioacetal (31a)

To a solution of (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal 30 (182 mg, 1 mmol) in acetone (30 mL) were added, anhydrous K_2CO_3 (553 mg, 4 mmol) and 1,3-dibromopropane (561 mg, 3 mmol) and the mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (10% EtOAc–hexane) to afford the compound **31a** as a white solid.

Yield 236 mg, 82%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.31–1.40 (m, 6H), 2.28–2.48 (m, 2H), 2.49–2.64 (m, 2H), 2.68–2.91 (m, 6H), 3.64 (m, 2H), 3.99 (s, 3H), 4.25 (t, *J* = 6.0 Hz, 2H), 4.56 (d, *J* = 6.7 Hz, 1H), 4.76 (m, 1H), 5.0–5.33 (m, 1H), 6.88 (s, 1H), 7.68 (s, 1H) LC MS: m/z 539.4 $[M]^+$. Anal. Calcd for $C_{20}H_{28}BrFN_2O_5S_2$: C, 44.53; H, 5.23; N, 5.19. Found: C, 44.43; H, 5.15; N, 5.10.

5.19.2. (2*S*)-*N*-[4-(5-Bromobutanoxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (31b)

The compound **31b** was prepared following the method described for the compound **31a**, by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal **30** (182 mg, 1 mmol) and 1,4-dibromobutane (605 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **31b** as white solid.

Yield 260 mg, 86%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.26–1.43 (m, 6H), 2.01–2.46 (m, 4H), 2.49–2.67 (m, 2H), 2.70–2.95 (m, 6H), 3.58 (m, 2H), 3.99 (s, 3H), 4.25 (t, *J* = 6.0, 2H), 4.55 (d, *J* = 6.7, 1H), 4.73–4.79 (m, 1H), 5.0–5.33 (m, 1H), 6.89 (s, 1H), 7.69 (s, 1H) LC MS: *m/z* 553 $[M]^+$. Anal. Calcd for $C_{21}H_{30}BrFN_2O_5S_2$: C, 45.57; H, 5.46; N, 5.06. Found: C, 45.40; H, 5.25; N, 4.95.

5.19.3. (2*S*)-*N*-[4-(6-Bromopentanoxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (31c)

The compound **31c** was prepared following the method described for the compound **31a**, by employing (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal **30** (182 mg, 1 mmol) and 1,5-dibromopentane (605 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **31c** as white solid.

Yield 220 mg, 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.26–1.41 (m, 6H), 1.74–2.0 (m, 4H), 2.35–2.47 (m, 2H), 2.49–2.65 (m, 2H), 2.70–2.92 (m, 6H), 3.58–3.66 (m, 2H), 3.98 (s, 3H), 4.25 (t, *J* = 6.0 Hz, 2H), 4.57 (d, *J* = 6.7 Hz, 1H), 4.75–4.85 (m, 1H), 5.0–5.34 (m, 1H), 6.88 (s, 1H). 7.70 (s, 1H) LC MS: *m/z* 567 $[M]^+$. Anal. Calcd for $C_{22}H_{32}BrFN_2O_5S_2$: C, 46.56; H, 5.68; N, 4.94. Found: C, 46.45; H, 5.55; N, 4.75.

5.20. General procedure for the synthesis of compounds (38a–c)

5.20.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitro-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (38a)

To a solution of (2*S*)-*N*-[4-(4-bromopropoxy)-5-methoxy-2-nitrobenzoyl]-4-fluoro pyrrolidine-2-carboxaldehyde diethylthioacetal (**31a**) (507 mg, 1 mmol) in dry acetonitril (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the piperazine (43 mg, 0.5 mmol). The reaction mixture was refluxed for 48 h. The reaction was monitored by TLC using (95% $CHCl_3$/CH_3OH) as a solvent system. The potassium carbonate was removed by suction filtration and the solvent was evaporated under vacuum. The crude product was purified by column chromatography (90% $CHCl_3$/CH_3OH) to afford the compound **38a** as a pale yellow solid.

Yield 845 mg, 80%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.29–1.36 (m, 12H), 2.02–2.11 (m, 4H), 2.46–2.69 (m, 8H), 2.73–2.89 (m, 12H), 3.39–3.62 (m, 4H), 3.98 (s, 6H), 4.12–4.25 (t, 4H), 4.52 (d, *J* = 6.79 Hz, 2H), 4.72 (q, *J* = 6.79 Hz, 2H), 5.02–5.15 (t, 4H), 5.20–5.29 (m, 2H), 6.84 (s, 2H), 7.65 (s, 2H) ESI MS: *m/z* 1003 $[M]^+$. Anal. Calcd for $C_{44}H_{64}F_2N_6O_{10}S_4$: C, 52.67; H, 6.43; N, 8.38. Found: C, 52.50; H, 6.35; N, 8.25.

5.20.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitro-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (38b)

The compound **38b** was prepared following the method described for the compound **38a**, employing piperazine and (2*S*)-*N*-[4-(5-bromobutanoxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrroli-

dine-2-carboxaldehyde diethylthioacetal (**31b**) (521 mg, 1 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **38b** as pale yellow solid.

Yield 860 mg, 85%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29–1.41 (m, 12H), 1.7–1.97 (m, 4H), 1.8–1.97 (m, 4H), 2.42–2.70 (m, 8H), 2.71–2.89 (m, 12H), 3.39–3.66 (m, 4H), 3.90–3.93 (s, 6H), 4.1–4.21 (t, 4H), 4.55 (d, J = 7.5 Hz, 2H), 4.70–4.81 (q, J = 6.79 Hz, 2H), 4.82–4.97 (m, 4H), 5.01–5.4 (m, 2H), 6.70 (s, 2H), 7.60 (s, 2H). Anal. Calcd for $C_{46}H_{68}F_2N_6O_{10}S_4$: C, 53.57; H, 6.65; N, 8.15. Found: C, 53.45; H, 6.50; N, 8.10.

5.20.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-nitro-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (38c)

The compound **37c** was prepared following the method described for the compound **37a**, employing piperazine and (2*S*)-*N*-[4-(6-bromopentanoxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (**31c**) (521 mg, 1 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **38c** as pale yellow solid (860 mg, 85%).

Yield 860 mg, 85%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.25–1.36 (m, 12H), 1.39–1.45 (m, 4H), 1.50–1.89 (m, 8H), 2.25–2.61 (m, 8H), 2.70–2.86 (m, 12H), 3.39–3.63 (m, 4H), 3.96 (s, 6H), 4.0 (t, 4H), 4.54 (d, J = 6.79 Hz, 2H), 4.75 (q, J = 6.0 Hz, 2H), 4.80–4.96 (m, 4H) 5.0–5.45 (m, 2H), 6.72 (s, 2H), 7.65 (s, 2H). Anal. Calcd for $C_{48}H_{72}F_2N_6O_{10}S_4$: C, 54.42; H, 6.85; N, 7.93. Found: C, 54.35; H, 5.65; N, 7.80.

5.21. General procedure for the synthesis of compounds (39a–c)

5.21.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (39a)

The compound **38a** (260 mg, 0.59 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 2.99 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 25 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **39a** as yellow liquid (210 mg, 88%), which was directly used in the next step due to potential stability problems.

5.21.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (39b)

The compound **39b** was prepared following the method described for the compound **39a**, employing the compound **38b** (967 mg, 1 mmol) to afford the amino diethylthioacetal **39b** as a yellow liquid (700 mg, 80%), which was directly used in the next step due to potential stability problems.

5.21.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4-fluoropyrrolidin-2-carboxaldehyde diethylthioacetal)] (39c)

The compound **39c** was prepared following the method described for the compound **39a**, employing the compound **38c** (967 mg, 1 mmol) to afford the amino diethylthioacetal **39c** as a yellow liquid (735 mg, 83%), which was directly used in the next step due to potential stability problems.

5.22. General procedure for the synthesis of compounds (14a–c)

5.22.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydro-piperazine] dioxy}-bis[(11a*S*)-2-fluoro-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (14a)

A solution of amino thioacetal **39a** (878 mg, 1 mmol), $HgCl_2$ (1.19 g, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in CH_3CN/H_2O (4:1) was stirred slowly at room temperature overnight until TLC indicated the complete disappearance of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a Celite. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (95% $CHCl_3$–CH_3OH) to afford the compound **14a** as a pale yellow solid (410 mg, 65%). This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

Yield 410 mg, 65%; $[\alpha]_{26}^{D}$ +164.0 (c 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.73–2.1 (m, 8H), 2.45–2.76 (m, 8H), 3.46–3.91 (m, 10H), 3.93 (s, 6H), 4.02–4.25 (m, 4H), 5.35–5.48 (d, 2H), 6.85 (s, 2H), 7.49 (s, 2H), 7.86 (d, J = 3.66 Hz, 2H) ESI MS: m/z 695 $[M+1]^+$. Anal. Calcd for $C_{36}H_{44}F_2N_6O_6$: C, 62.23; H, 6.38; N, 12.10. Found: C, 62.10; H, 6.25; N, 12.0.

5.22.2. 1,1′-{[1,4-Di(butane-1,4-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-2-fluoro-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (14b)

The compound **14b** was prepared following the method described for the compound **14a**, employing the compound **39b** (967 mg, 1 mmol) to afford the compound **14b** as a pale yellow solid.

Yield 350 mg, 62%; $[\alpha]_{26}^{D}$ +174.0 (c 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.26–1.94 (m, 8H), 2.11–2.47 (m, 4H), 2.59–2.75 (m, 8H), 3.48–3.86 (m, 10H), 3.93 (s, 6H), 4.0–4.25 (m, 4H), 5.27–5.55 (m, 2H), 6.82 (s, 2H), 7.49 (s, 2H), 7.85 (d, J = 4.4 Hz, 2H). Anal. Calcd for $C_{38}H_{48}F_2N_6O_6$: C, 63.14; H, 6.69; N, 11.63. Found: C, 63.10; H, 6.55; N, 11.52.

5.22.3. 1,1′-{[1,4-Di(pentane-1,5-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-2-fluoro-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (14c)

The compound **14c** was prepared following the method described for the compound **14a**, employing the compound **39c** (967 mg, 1 mmol) to afford the compound **14c** as a pale yellow solid.

Yield 750 mg, 46%; $[\alpha]_{26}^{D}$ +88.0 (c 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.44–2.09 (m, 12H), 2.30–2.51 (m, 4H), 2.52–3.0 (m, 8H), 3.47–3.87 (m, 10H), 3.93 (s, 6H), 4.0–4.1 (m, 4H), 5.27–5.58 (m, 2H), 6.9 (s, 2H), 7.49 (s, 2H), 7.9 (d, 2H, J = 4.6 Hz). Anal. Calcd for $C_{40}H_{52}F_2N_6O_6$: C, 63.98; H, 6.98; N, 11.19. Found: C, 63.90; H, 6.88; N, 11.10.

5.23. General procedure for the synthesis of compounds (33)

5.23.1. (2*S*)-*N*-[4-(4-Bromopropoxy)-5-methoxy-2-nitrobenzoyl]-4-difluoromethylidenepyrrolidine-2-carboxaldehyde diethylthioacetal (33)

To a solution of (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)-4-difluoromethylidine pyrrolidine-2-carboxaldehyde diethylthioacetal **32** (182 mg, 1 mmol) in acetone (30 mL) were added, anhydrous K_2CO_3 (553 mg, 4 mmol) and 1,3-dibromopropane (561 mg, 3 mmol) and the mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC. After completion of the

reaction, potassium carbonate was removed by filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (10% EtOAc–hexane) to afford the compound **33** as a white solid.

Yield 236 mg, 82%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.15–1.40 (m, 6H), 2.41–2.47 (m, 2H), 2.69–2.97 (m, 6H), 3.59–3.67 (t, J = 6.04 Hz, 2H), 3.78–3.82 (m, 2H), 3.97 (s, 3H), 4.25–4.29 (t, J = 5.28 Hz, 2H), 4.63 (d, J = 3.0 Hz, 1H), 4.96–4.98 (m, 1H), 6.82 (s, 1H), 7.76 (s, 1H). ESI MS m/z 607 $[M+K]^+$. Anal. Calcd for $C_{21}H_{27}BrF_2N_2O_5S_2$: C, 44.29; H, 4.78; N, 4.92. Found: C, 44.15; H, 4.64; N, 4.80.

5.24. General procedure for the synthesis of compounds (40)

5.24.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*7-methoxy-2-nitro-benzoyl-(4-difluoromethylidinepyrrolidin-2-carboxaldehydediethylthioacetal)] (40)

To a solution of (2*S*)-*N*-[4-(4-bromopropoxy)-5-methoxy-2-nitrobenzoyl]-4-difluoro methylidinepyrrolidine-2-carboxaldehyde diethylthioacetal (**33**) (507 mg, 1 mmol) in dry acetonitril (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the piperazine (43 mg, 0.5 mmol). The reaction mixture was refluxed for 48 h. The reaction was monitored by TLC using ethyl acetate–hexane (8:2) as a solvent system. The potassium carbonate was removed by suction filtration and the solvent was evaporated under vacuum. The crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **40** as a pale yellow solid.

Yield 845 mg, 90%; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.19–1.42 (m, 12H), 1.61–2.0 (m, 4H), 2.56–2.65 (m, 8H), 2.68–2.96 (m, 12H), 3.56–3.87 (m, 8H), 3.98 (s, 6H), 4.19 (t, 4H,), 4.59 (d, J = 3.77 Hz, 2H), 4.91–4.94 (m, 2H), 6.76 (s, 2H), 7.72 (s, 2H). ESI MS m/z 1063 $[M+1]^+$. Anal. Calcd for $C_{46}H_{62}F_4N_6O_{10}S_4$: C, 51.96; H, 5.88; N, 7.90. Found: C, 51.75; H, 5.65; N, 7.80.

5.25. General procedure for the synthesis of compounds (41)

5.25.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-7-methoxy-2-amino-benzoyl-(4-difluoromethylidinepyrrolidin-2-carboxaldehyde diethylthioacetal)] (41)

The compound **40** (260 mg, 0.59 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 2.99 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 25 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **41** as yellow liquid (210 mg, 88%), which was directly used in the next step due to potential stability problems.

5.26. General procedure for the synthesis of compounds (15)

5.26.1. 1,1′-{[1,4-Di(propane-1,3-diyl)hexahydropiperazine] dioxy}-bis[(11a*S*)-2-difluoromethylidine-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one] (15)

A solution of amino thioacetal **41** (878 mg, 1 mmol), $HgCl_2$ (1.19 g, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in CH_3CN/H_2O (4:1) was stirred slowly at room temperature overnight until TLC indicated the complete disappearance of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a Celite. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (95% $CHCl_3$–CH_3OH) to afford the compound **15** as a pale yellow solid this material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine for.

Yield 410 mg, 65%; $[\alpha]_{26}^{D}$ +86.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.0–2.1 (m, 4H), 2.54–2.79 (m, 8H), 2.96–3.14 (m, 4H), 3.56–3.83 (m, 8H), 3.97 (m, 6H), 4.0–4.1 (m, 4H), 4.33–4.34 (t, 2H), 6.82 (s, 2H), 7.47 (s, 2H), 7.68 (d, J = 3.67 Hz, 2H). ESI MS m/z 755 $[M+1]^+$. Anal. Calcd for $C_{38}H_{42}F_4N_6O_6$: C, 60.47; H, 5.61; N, 11.13. Found: C, 60.30; H, 5.55; N, 11.05.

5.27. Biological activity

5.27.1. Thermal denaturation assay

The compound **8a–c**, **13a–c**, **14a–c** and **15** were subjected to DNA thermal melting (denaturation) studies using duplex form calf thymus DNA (CT-DNA) using modification reported procedure.[41] Working solutions were produced by appropriate dilution in aqueous buffer (10 mM) Na_2HPO_4/NaH_2PO_4, 1 mM Na_2EDTA, pH 7.00 + 0.01) containing CT-DNA (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in MeOH to obtain a fixed [PBD]/[DNA] molar ratio of 1:5 the DNA-PBD solutions were incubated at 37 °C for 0 h prior to analysis sample were monitored a 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller. Heating was applied at a rate of 1 °C/min in the 40–90 °C temperature range. DNA-helix-coil transition temperature (T_m) were determined from the maxima in the (dA 260)/dT derivative plots. Results for each compound are shown as mean ± standard derivation from the least three determination and are corrected for the effects of MeOH cosolvent using a linear correction term.[42] Ligand-induced alteration in DNA melting behavior (ΔT_m) are given by $\Delta T_m = T_m$ (DNA + PBD) $- T_m$ (DNA alone), were the T_m value for the PBD free CT-DNA is 69.2 ± 0.001 the fixed [PBD]/[DNA] ratio used did not results in binding saturation of the host DNA duplex for any compound examined.

5.27.2. Restriction endonuclease inhibition

Stock solutions of each PBD (100 μM) were prepared by dissolving each compound in DMSO (Sigma). These were stored at −20 °C. Plasmid (pBR322) containing single *Bam*H1site was used in this assay. Restriction endonuclease and the relevant buffer were obtained from NEB. The DNA fragment (500 mg) was incubated with each PBD (see Fig. 2 for PBD concentrations) in a final volume of 16 μM for 16 h at 37 °C. Next 10 × *Bam*H1 buffer (2 μM) was added, and the reaction mixture was made to 20 μM with *Bam*H1 (20 units) and then incubated for 1 h at 37 °C. Then loaded on to a 1% agarose gel electrophoresis in tris acetate EDTA buffer at 80 V for 2 h. The gels were stained with ethidium bromide and photographed.

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